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FORMATION AND DISPOSITION OF 7-HYDROXYMETHOTREXATE IN RABBITS

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Abstract—The metabolism of methotrexate in rabbits was investigated following 6 hr of infusion with [3′,5′,7-^{3}H]methotrexate (50 mg/kg). Methotrexate and its metabolites were analyzed by an enzyme kinetic method, reverse phase high-performance liquid chromatography, and scintillation counting of radioactivity. 7-Hydroxymethotrexate was found to be a major metabolite in plasma, urine, bile and various tissues of rabbits. Cumulative production of 7-hydroxymethotrexate during the first 6 hr was 31.8% of the total dose. The peak 7-hydroxymethotrexate concentration in plasma was reached at the end of the infusion, and this concentration was higher than the methotrexate concentration. The plasma clearance of 7-hydroxymethotrexate was biphasic in nature and slower than that of methotrexate. The highest metotrexate concentration was found in kidney after 6 hr. This concentration was found to be 7.2 times greater than that in plasma. A higher ratio of 7-hydroxymethotrexate/methotrexate was found in liver, small intestine, kidney and testis when compared to plasma. Lung and kidney showed significant conversion of methotrexate to 7-hydroxymethotrexate *in vitro*, as did the liver.

On the basis of early clinical pharmacologic studies [1, 2], metabolism of methotrexate (2,4-diamino-N^{10}-methylpteroylglutamic acid; MTX) was thought to be negligible. A few years later, MTX metabolites were detected in urine, blood, feces and various tissues after high dose treatment [3]. Since then, the major metabolites that have been identified in human samples are 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) [3, 4], MTX polyglutamates [5, 6], and 7-hydroxymethotrexate (7-OH-MTX) [7], particularly after high dose MTX therapy [8, 9].

In humans, a higher percentage of APA production has been reported after oral MTX administration than after systemic infusion [10], but its pharmacologic role in humans is still unclear. Intestinal flora are responsible for the conversion of MTX to APA [3, 4]. Methotrexate polyglutamates have been detected in human erythrocytes [7] and liver tissue [8]. The polyglutamates, like MTX, inhibit dihydrofolate reductase (DHFR) activity, but the effects of MTX polyglutamates on the human cell remain unknown. 7-Hydroxymethotrexate is the major metabolite of MTX that has been identified in urine and plasma after high dose therapy and may contribute to renal toxicity because of its poor solubility [9]. The DHFR inhibitory activity of 7-OH-MTX, however, is two orders of magnitude less than that of MTX [11–13]. The rabbit possesses an ability to convert MTX to 7-OH-MTX and can tolerate high dose MTX infusion without any rescue therapy [14]. In the present study, we have investigated the pharmacodynamics of MTX, concentrating on the biosynthesis and distribution of 7-OH-MTX in rabbits after high dose infusion of tritiated methotrexate.

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MATERIALS AND METHODS

Male New Zealand white rabbits weighing 3.7 to 4.0 kg were used. Methotrexate was obtained from the National Cancer Institute, Division of Cancer Treatment, Bethesda, MD. [3′,5′,7-^{3}H]Methotrexate sodium salt (15.8 Ci/mmole) was obtained from the Amersham Corp., Arlington Heights, IL. Methotrexate and [3′,5′,7-^{3}H]MTX were purified by DEAE-cellulose column chromatography prior to their use [12]. 7-Hydroxymethotrexate was a gift from Dr. David Farquhar, Department of Developmental Therapeutics, University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, TX; MTX polyglutamates were provided by Dr. Charles M. Baugh, University of Southern Alabama, Mobile, AL. The APA was prepared by treating MTX with carboxypeptidase G [15]. Hexanesulfonic acid sodium salt was obtained from the Eastman Organic Chemical Co., Rochester, NY. Glass-distilled methanol was obtained from the Burdick & Jackson Laboratories, Muskegon, MI.

Fifty milligrams of [3′,5′,7-^{3}H]MTX per kilogram of body weight (9.16 to 16.50 μCi/kg) was infused into an ear vein for 6 hr. Blood samples were obtained from a vein of the other ear; urine and feces were collected separately. Bile was obtained, from a catheter fixed in the common bile duct, before and after MTX infusion. Rabbits were killed in a CO_2 gas chamber at 6, 20, and 48 hr after the start of infusion. The organs were immediately removed and stored at −70° until analysis. The concentrations of MTX and its metabolites were assayed by an enzyme kinetic method [16] and by high-performance liquid chromatography [17], and aliquots that

were collected from HPLC analysis were subsequently counted on a Beckman LS-7500 scintillation counter for verification.

For HPLC analysis, serum, bile, and extracts of tissue homogenates were prepared by the following procedures. The samples were boiled for 5 min to denature proteins. The supernatant fluid of the boiled sample was extracted with 2 ml of methanol in a prewetted C18 SEP PAK Cartridge (Waters Associates Inc., Milford, MA). The methanol extract was evaporated under nitrogen gas, and the dried residue was then dissolved in 200 μl of distilled water. Sample extracts (10–50 μl) were injected into the HPLC system. The percentage recoveries of 7-OH-MTX, MTX and APA from the serum during the extraction were 38.5 ± 5.8, 45.5 ± 7.2 and $43.4 \pm 14.1\%$ respectively. The percentage recoveries of the MTX polyglutamates, $MTXG_1$ and $MTXG_2$, were 38.6 ± 16.5 and $32.1 \pm 22.4\%$, respectively, when these compounds were dissolved in distilled water. The recovery studies were performed in the concentration range of 10^{-7} to 10^{-4} M.

A Glenco HPLC system (Glenco Instrument, Houston, TX) with a 5 μm C18, Spherisorb ODS 4.6 mm × 150 mm column (Custom LC, Inc., Houston, TX) was used. The system was equipped with a variable wavelength u.v. detector (LDC Spectromonitor III: Laboratory Data Control, Riviera Beach, FL) and an electronic integrator, model CSI-38 (Columbia Scientific Industries, Austin, TX). The mobile phase was a solution of 70% (v/v) 5 mM hexanesulfonic acid, pH 3.75, and 30% methanol. The flow rate was 1 ml/min, and u.v. absorbance was monitored at 305 nm. The details of this method have been published [17]. In the present study, however, a different reverse phase column was used. The retention times of 7-OH-MTX and MTX were 14.3 ± 0.4 and 31.3 ± 0.8 min respectively.

The conversion of MTX to 7-OH-MTX in tissues was determined *in vitro* by the incubation of 10^{-4} M [3′,5′,7-^{3}H]MTX with liver, kidney, lung, brain, or muscle homogenate at 37° for 10 min to 20 hr. Tissue homogenates were prepared after adding 3 vol. of 10 mM Tris–HCl buffer (pH 7.6) that included 10 mM $MgCl_2$ and 0.25 M sucrose. After the incubation period, the samples were put in a 100° waterbath, and the supernatant fluids were extracted as previously outlined. Methotrexate and its metabolites were measured by the HPLC method, followed by the counting of collected HPLC eluates.

RESULTS

7-Hydroxymethotrexate was found to be a major metabolite in the plasma, urine, bile, and pooled tissues of rabbits. Cumulative production of 7-OH-MTX during 6 hr of infusion was 31.7% of the total dose; its distribution was 14.7% in the pooled tissues, 8.5% in excreted urine, 6.8% in the contents of the alimentary tract, and 1.7% in bile (Table 1). At 20 hr after the start of infusion, the cumulative production of 7-OH-MTX was 16.8% distributed as 5.3% in the pooled tissues, 9.9% in the urine, 0.6% in the contents of the alimentary tract, and 1.0% in bile. At 48 hr, the cumulative production of 7-OH-MTX was 20.6%, with 0.9% in tissues, 12.1% in urine, 2.1% in the alimentary tract, and 5.5% in feces.

Total radioactivity, and the MTX and 7-OH-MTX

Table 1. Percentage distribution of MTX and 7-OH-MTX radioactivities in rabbits after [^{3}H]MTX (150 mg/kg) infusion

Tissue or fluids		Distribution of radioactivities (%)		
		Hours from the start of infusion		
		6	20	48
Rabbit tissues*	Total radioactivity (TR)	42.1	10.6	6.2
	MTX	12.3	1.6	1.4
	7-OH-MTX	14.7	5.3	0.9
GI tract	TR	26.3	1.1	3.0
	MTX	19.4	0.2	0.1
	7-OH-MTX	6.8	0.6	2.1
Bile	TR	2.6	1.8	†
	MTX	0.6	0.4	†
	7-OH-MTX	1.7	1.0	†
Feces	TR	‡	†	8.6
	MTX	‡	†	0.5
	7-OH-MTX	‡	†	5.5
Urine	TR	28.6	57.1	65.5
	MTX	14.4	45.1	40.5
	7-OH-MTX	8.5	9.9	12.1
Total recovery	TR	99.6	70.9§	85.2
	MTX	46.7	47.4§	42.5
	7-OH-MTX	31.7	16.8§	20.6

* Rabbit tissues used were listed in Table 2.

† Samples were not collected due to technical difficulties. During the first 20 hr, all rabbits were in a special restraining box where bile fluids were collected.

‡ No feces were produced.

§ These numbers exclude those of feces.

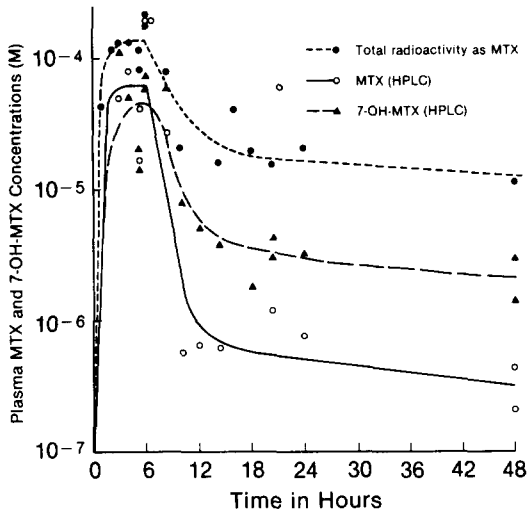

Fig. 1. Plasma MTX and 7-OH-MTX concentrations in rabbits infused with [^{3}H]MTX (50 mg/kg). Total radioactivity (●- - -●) in plasma was calculated as MTX equivalents. MTX (○———○) and 7-OH-MTX (▲- - -▲) concentrations were assayed by HPLC. The lines were computer generated [18].

concentrations of plasma, reached peak levels at the end of the 6-hr infusion. The peak concentrations of total radioactivity, MTX, and 7-OH-MTX were 1.8×10^{-4}, 1.6×10^{-4}, and 6.0×10^{-5} M respectively. Total radioactivity as MTX equivalent, and MTX and 7-OH-MTX concentrations after the MTX infusion, were analyzed by the method of nonlinear estimation of variables (NONLIN) [18]. The half-lives of total radioactivity were 1.4 hr (α) and 541.6 hr (β) (correlation 0.993), MTX were 0.7 hr (α) and 47.7 hr (β) (correlation 1.000) and of 7-OH-MTX were 2.0 hr (α) and 105.6 hr (β) (correlation 1.000) (Fig. 1).

The highest radioactivity per gram wet tissue was found in the kidney at the completion of MTX infusion and was 7.2 times higher than that in plasma. The values for radioactivity in liver, lung, brain, small intestine, testis, and fat were similar to that of plasma. However, the values of radioactivity in skeletal muscle, heart, and spleen were less than 50% of the plasma level. Only 7-OH-MTX was found in the liver and small intestine; no MTX was detected. Lung and testis had high 7-OH-MTX/MTX ratios, being 19.2 and 4.6 respectively. These high ratios of 7-OH-MTX/MTX persisted for 20 and 48 hr. In the kidney, however, the 7-OH-MTX/MTX ratio

Table 2. MTX and 7-OH-MTX concentrations in rabbit tissues after [^{3}H]MTX infusion (50 mg/kg)

	Concentrations of MTX and 7-OH-MTX (μM)					
	6 Hr*		20 Hr		48 Hr	
	RA†	HPLC	RA	HPLC	RA	HPLC
		MTX (a) 7-OH-MTX (b)		MTX (a) 7-OH-MTX (b)		MTX (a) 7-OH-MTX (b)
Kidney	1420.0	523.0 (a) 380.0 (b)	42.0	ND‡ (a) 21.2 (b)	35.6	ND (a) 15.0 (b)
Liver	117.0	ND (a) 127.0 (b)	31.0	ND (a) 38.9 (b)	53.0	ND (a) 33.9 (b)
Lung	114.0	4.3 (a) 82.0 (b)	59.3	4.8 (a) 36.4 (b)	18.3	ND (a) 9.2 (b)
Brain	254.0	40.5 (a) 69.1 (b)	11.9	2.2 (a) 17.0 (b)	10.8	0.2 (a) 0.7 (b)
Testis	187.0	13.0 (a) 59.2 (b)	14.7	ND (a) 40.2 (b)	5.3	0.2 (a) 3.0 (b)
Spleen	84.3	48.4 (a) 45.8 (b)	26.6	8.0 (a) 14.6 (b)	9.7	ND (a) ND (a)
Heart muscle	46.4	54.0 (a) 43.3 (b)	9.7	2.6 (a) 5.2 (b)	ND ND	ND (a) ND (b)
Small intestine	338.0	ND (a) 58.2 (b)	16.7	ND (a) 14.5 (b)	16.1	ND (a) 1.8 (b)
Fat tissue	243.0	26.0 (a) 24.5 (b)	2.0	ND (a) ND (b)	1.8	ND (a) ND (b)
Muscle	64.9	16.9 (a) 5.5 (b)	28.7	13.0 (a) 19.0 (b)	22.6	9.7 (a) 2.1 (b)
Plasma	196.0	158.0 (a) 59.5 (b)	20.2	1.2 (a) 3.0 (b)	11.4	0.2 (a) 3.0 (b)

* Hours from the start of infusion.
† Total radioactivity as MTX equivalent.
‡ Not detectable by HPLC.

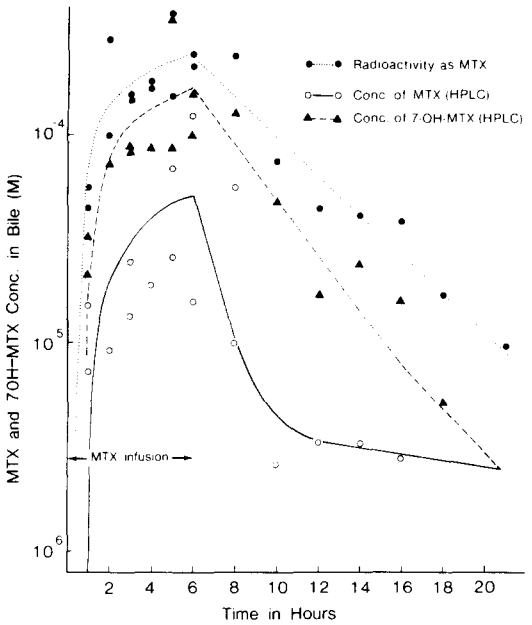

Fig. 2. Biliary MTX and 7-OH-MTX concentrations in rabbits infused with [^{3}H]MTX (50 mg/kg). Bile was collected from the catheter fixed on the common bile duct before MTX infusion. Total radioactivity (●- - -●) was calculated as MTX equivalents. MTX (○———○) and 7-OH-MTX concentrations (▲- - -▲) were assayed by HPLC.

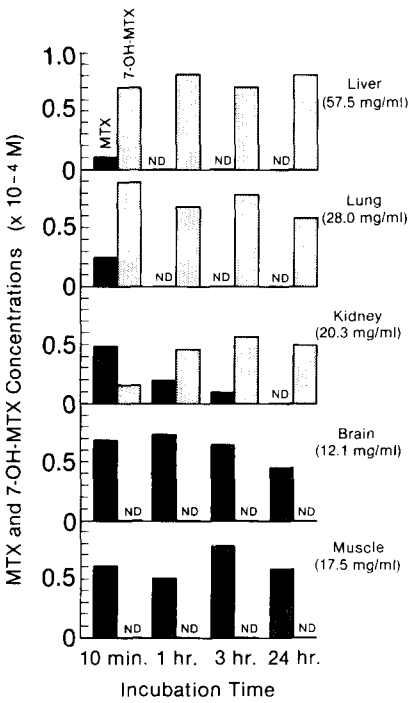

Fig. 3. Conversion of MTX (10^{-4} M) to 7-OH-MTX in rabbit tissue homogenates. The homogenates of rabbit liver (protein concentration, 57.5 mg/ml), lung (28.0 mg/ml), kidney (20.3 mg/ml), brain (12.1 mg/ml), and muscle (17.5 mg/ml) were incubated with 10^{-4} M MTX. MTX and 7-OH-MTX concentrations of incubation mixtures were measured by HPLC from 10 min to 24 hr. ND = not detectable.

was 0.7 at 6 hr, but only 7-OH-MTX was detected at 20 and 48 hr. Methotrexate polyglutamates and APA were not found by HPLC, and no appreciable radioactivity could be traced by counting the collected aliquots.

Table 2 shows the concentrations of MTX and 7-OH-MTX in the various tissues and plasma. Elimination of the drug from lung and brain was similar to that from plasma, but elimination of MTX and 7-OH-MTX from liver, kidney, and muscle was slower than that from plasma.

Figure 2 shows biliary MTX and 7-OH-MTX concentrations. Total radioactivity, MTX, and 7-OH-MTX levels reached peaks at 6 hr (duration of infusion); the peak concentration for total radioactivity was 2.7×10^{-4} M; for MTX, 7.1×10^{-5} M; and for 7-OH-MTX, 2.4×10^{-4} M. After the infusion, total radioactivity and MTX and 7-OH-MTX concentrations rapidly cleared with a one-phase pattern for total radioactivity and 7-OH-MTX and a biphasic pattern (α, β) for total MTX. The half-lives of the total radioactivity and 7-OH-MTX were 3.3 and 2.3 hr respectively. The half-lives of MTX, calculated by NONLIN, were 0.06 hr (α) and 26.8 hr (β) (correlation 0.901).

In vitro tissue conversion of MTX to 7-OH-MTX is shown in Fig. 3. Methotrexate was rapidly metabolized to 7-OH-MTX in liver (protein concentration, 57.5 mg/ml) and lung (28.0 mg/ml), and metabolism was completed within 1 hr. Kidney (protein concentration, 20.3 mg/ml) had moderate activity, and conversion was completed by 4 hr. Brain (protein concentration, 12.1 mg/ml) and muscle (17.5 mg/ml) showed no conversion. The activities of conversion to 7-OH-MTX calculated during the first 10 min were 2.89×10^{-10} moles per mg protein per min in liver, 7.02×10^{-10} moles per mg per min in lung, and 1.70×10^{-10} moles per mg per min in kidney. The Michaelis–Menten constant for 7-OH-MTX production from [^{3}H]MTX by rabbit liver crude homogenate was 1.25 mM.

DISCUSSION

MTX was rapidly converted to 7-OH-MTX, up to 30% of the administered dose being converted during the infusion period (6 hr). The metabolism of MTX to 7-OH-MTX decreased after the MTX infusion, because a large quantity of administered MTX was distributed in muscle tissue that showed negligible conversion of MTX.

The comparison of MTX and 7-OH-MTX levels in rabbit tissues taken at different times revealed that MTX was eliminated more rapidly from the tissues than was 7-OH-MTX over the first 20 hr. After 20 hr, however, the tissue content of MTX remained relatively stable, being about 1.5% of the dose administered. This small amount of MTX or its metabolites was considered to be tightly bound to tissue proteins. 7-Hydroxymethotrexate was constantly eliminated from the rabbit tissues for up to 48 hr. The continued elimination of 7-OH-MTX is

attributed to the weak capacity for binding of 7-OH-MTX to tissue proteins [12].

The main excretion route of MTX and 7-OH-MTX was the urinary tract. Sixty-five percent of the administered radioactivity was excreted in the urine in 48 hr, 40.5% as MTX, and 12.1% as 7-OH-MTX. The renal clearance of 7-OH-MTX is thought to be slower than that of MTX; the poor water solubility of 7-OH-MTX [9] most likely accounts for its slow renal clearance.

In feces, only 0.5% of the administered MTX was excreted as intact MTX, but 5.5% was excreted as 7-OH-MTX over 48 hr. This value represents one-fourth of the total 7-OH-MTX produced in rabbits. 7-Hydroxymethotrexate appeared to be excreted more effectively in feces than was MTX, since the concentration of 7-OH-MTX was consistently higher than MTX in the bile, and the reabsorption of 7-OH-MTX (20.3%) was much less than that of MTX (97.7%). These results are derived from a comparison of the MTX and 7-OH-MTX contents of the alimentary tract, at the end of MTX infusion, and from the total excretion of these materials in feces up to 48 hr.

Johns *et al.* [19] identified the MTX-inactivating enzyme in the rabbit as aldehyde oxidase and have shown it to be localized in rabbit liver. In the rabbit, higher ratios of 7-OH-MTX/MTX, compared to that in plasma, were found not only in liver but also in lung, small intestine, kidney, and testis. Both lung and kidney also showed MTX to 7-OH-MTX conversion that was similar in magnitude to that in liver *in vitro*. The conversion of MTX to 7-OH-MTX in these various tissues is considered to be catalyzed by an enzyme similar to that in the liver. Aldehyde oxidase is assumed to be located not only in the liver but also in the lung, kidney, and other tissues that had high ratios of 7-OH-MTX/MTX. The rabbit tissues that had high conversion of MTX to 7-OH-MTX are considered to be protected from the toxicity of MTX since 7-OH-MTX has much less inhibitory activity toward dihydrofolate reductase [13]. In fact, to maintain a level of 10^{-4} M MTX in rabbit liver homogenate for an hour, more than 10^{-3} M of initial MTX concentration is necessary in the incubation medium.

The report by Johns and Valerino [12] indicated that aldehyde oxidase has an extremely wide range of species variation. In human liver, despite the fact that cumulative 7-OH-MTX recovery was up to 10% of administered MTX with high dose therapy [9], the aldehyde oxidase activity with MTX as the substrate was negligible on spectrophotometric analysis [12]. Therefore, it is possible that aldehyde oxidase is also widely distributed in humans. Further investigation of the distribution of the enzyme and detailed pharmacologic analysis of 7-OH-MTX in humans will be important in understanding the mechanism of toxicity or of resistance to high dose MTX therapy in primary and metastatic tumor tissues.

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